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IDENTIFICATION AND SYNTHESIS OF TRINORCADALENE PHYTOALEXINS FORMED BY *HIBISCUS CANNABINUS*

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Hibiscus cannabinus*; *Gossypium* species; Malvaceae; kenaf; cotton; *Verticillium dahliae*; *Fusarium oxysporum*; wilt diseases; phytoalexins; disease resistance; terpenoids; trinorcadalenes.

Abstract—Two trinorcadalene phytoalexins, hibiscanal (2,8-dihydroxy-4,7-dimethoxy-6-methyl-1-naphthaldehyde) and *o*-hibiscanone (3,8-dimethyl-1,2-naphthoquinone), were isolated and identified from stem stele of kenaf (*Hibiscus cannabinus*) inoculated with the fungal pathogen *Verticillium dahliae*. *o*-Hibiscanone also was synthesized. The ED_{50} values of hibiscanal and *o*-hibiscanone against *V. dahliae* were 25.83 and 1.18 μg/ml, respectively. *o*-Hibiscanone killed all propagules of *V. dahliae* and *Fusarium oxysporum* f. sp. *vasinfectum* at 8 μg/ml and 12 μg/ml, respectively. *o*-Hibiscanone is more toxic to *V. dahliae* than desoxyhemigossypol, the most toxic phytoalexin known in cotton (*Gossypium* species), or mansonone C (3,8-dimethyl-5-isopropyl-1,2-naphthoquinone) which has been isolated from other malvaceous species. The implications of these findings for the genetic engineering of cotton phytoalexins is discussed.

INTRODUCTION

A number of phytoalexins (antifungal antibiotics synthesized by plants in response to microbial infections) have been isolated and identified from cotton plants (*Gossypium* species, tribe Gossypieae, family Malvaceae) infected with *Verticillium dahliae* Kleb. The most prominent phytoalexins in vascular tissues of cultivated Upland cotton (*Gossypium hirsutum* L.) are the cadalene terpenoids hemigossypol (HG) and desoxyhemigossypol (dHG) which are accompanied by relatively low concentrations of their 6-methyl ethers, hemigossypol-6-methyl ether (MHG) and desoxyhemigossypol-6-methyl ether (dMHG) [1–3] (Scheme 1). High concentrations of the methyl ethers are found in cultivated Pima and Egyptian cottons (*Gossypium barbadense* L.) and in several wild *Gossypium* species, which may contain more than 50% of the phytoalexins as the methyl ethers [4, 5]. Raimondal (2-methoxy-hemigossypol); occurs uniquely in the wild species *Gossypium raimondii* Ulbr. [6].

Cotton cultivars that are resistant to Verticillium wilt and Fusarium wilt accumulate phytoalexins in infected xylem vessels more rapidly than do susceptible cultivars [3, 7–10]. Accumulation of the phytoalexins is correlated closely with induction of the genes coding for 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) and δ-cadinene synthase (CADS) [8, 11–13]. In the most resistant cultivars, maximum accumulation of mRNA from these genes is observed by 12 hr after inoculation, whereas accumulation is delayed by 24 to 48 hr in susceptible cultivars. Most modern cultivars of Upland cotton that have been developed for resistance to Verticillium wilt exhibit rapid accumulation of terpenoid aldehydes and rapid induction of HMGR and CADS genes, apparently due to transfer of these characters from wilt-resistant *G. barbadense* cultivars [8]. It is unlikely that this character can be improved much further because maximum accumulation of mRNA for HMGR and CADS occurs by 12 hr after inoculation, which is even before the conidia of *V. dahliae* normally germinate. Likewise, the induction of these genes is so sensitive in the resistant cultivars that phytoalexin synthesis is triggered by dead as well as by live conidia of the fungus [3, 14].

Further progress in improving the phytoalexin response of cotton might be achieved by increasing the potency of the phytoalexins produced. The phyto-

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Hemigossypol R = H
Hemigossypol-6-methyl ether R = CH_3
Desoxyhemigossypol R = H
Desoxyhemigossypol-6-methyl ether R = CH_3

Scheme 1. Terpenoid phytoalexins of *Gossypium*.

alexins MHG and dMHG, and raimondal, are only about one-half as toxic as the nonmethylated parent compounds to several plant and human pathogens [5, 15–18]. Accordingly, the evolutionarily advanced *Gossypium* species, *G. hirsutum*, *G. arboreum* L., and *G. herbaceum* L. [19], have evolved regulatory genes to restrict the methylation of desHG and, thus, decrease the percentage of the phytoalexins that are methylated [5, 20]. Further, when genes were introduced into Upland cotton from other *Gossypium* species to increase the percentages of the less potent phytoalexins MHG and dMHG or raimondal among total phytoalexins, there was a concomitant decrease in resistance to Verticillium wilt in each of four family lines. Thus, the low potency of cotton phytoalexins appears to limit their effectiveness against wilt pathogens.

Kenaf (*Hibiscus cannabinus* L.), an annual fiber crop used for the manufacture of newsprint, might be the source of genes that could be used to improve the potency of cotton phytoalexins. This species, like cotton, belongs to the *Malvaceae* family. It was the most resistant of any dicotyledonous species to isolates of *Verticillium dahliae* from 17 species belonging to 11 plant families in Russia [21]. Four kenaf cultivars also showed high levels of resistance to both defoliating and nondefoliating strains of *V. dahliae* isolated from cotton in the United States [22]. In this paper we report the isolation and biological activity of two trinorcadalene phytoalexins induced by *V. dahliae* infection in stele tissues of kenaf plants. A synthesis is reported for the more potent phytoalexin, *o*-hibiscanone.

RESULTS

Isolation of phytoalexins from kenaf

TLC plate bioassays of the crude extracts of kenaf stem steles inoculated with *V. dahliae* clearly showed two spots inhibitory to *V. dahliae* and *Fusarium oxysporum* Schlecht f. sp. *vasinfectum* Atk. Sny. & Hans. (*F.o.v.*). One spot was extremely inhibitory to both pathogens. No antifungal activities were observed from extracts of healthy stem stele.

Table 1. Rf values of *o*-hibiscanone and hibiscanal in different solvent systems

Solvent Systems	*o*-Hibiscanone	Hibiscanal
Cyclohexane : EtOAc (60 : 40)	0.49	0.10
Toluene : MeOH (95 : 5)	0.53	0.03
Hexane : Me_2CO (60 : 40)	0.53	0.18
$CHCl_3$: Me_2CO : HCO_2H (85 : 15 : 1)	0.69	0.33

Column chromatography was used to isolate the inhibitory compounds demonstrated by the TLC plate bioassay. Antifungal activity was found in column fractions 3 and 5. Chromatography of these fractions on TLC plates and crystallization of the eluted compounds were used to purify the inhibitory compounds, which were identified as *o*-hibiscanone and hibiscanal. R_f values of these compounds on TLC plates in various solvents is shown in Table 1.

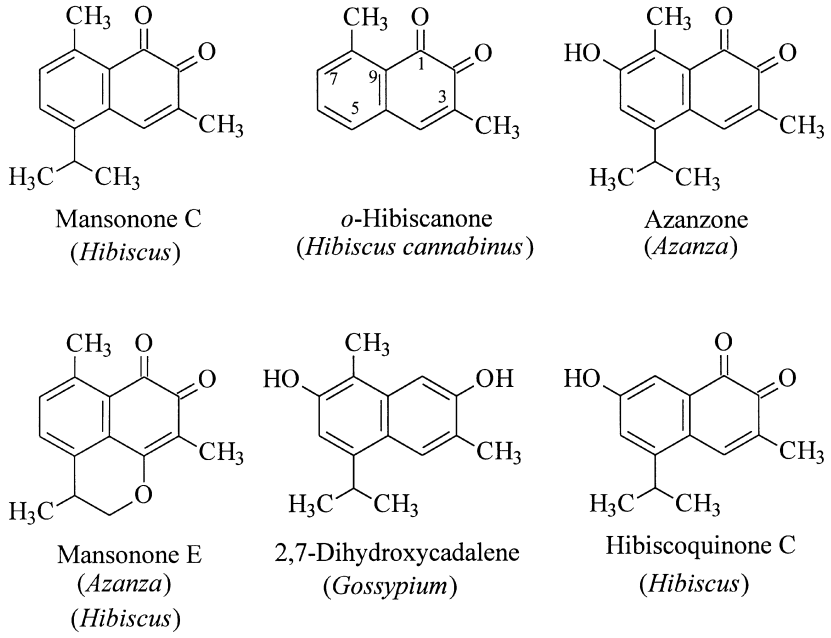
On developed TLC plates, hibiscanal appeared as a yellow spot, and *o*-hibiscanone as a yellow-orange spot. A dark-blue color appeared when the compounds were sprayed with ferric chloride-potassium ferricyanide. With phloroglucinol spray, hibiscanal initially gave a brown color that turned to orange after several minutes.

Identification of o-hibiscanone

o-Hibiscanone was identified as 3,8-dimethyl-1,2-naphthoquinone (Scheme 2) by x-ray crystallography. Its ^{1}H- and ^{13}C-NMR and mass spectra are given in the Experimental Section and it was synthesized as shown in Fig. 1.

Identification of hibiscanal

Hibiscanal has the structure shown in Scheme 3. It was obtained as orange crystals, mp 225–226°, from acetone, and gave a parent peak in the mass spectrum at *m/z* 262 (7.6%). High resolution mass measurement gave the formula $C_{14}H_{14}O_5$ (Calcd. 262.0841; Found



Scheme 2. Comparison of *o*-hibiscanone structure with those of related terpenoids in other species of the Malvaceae.

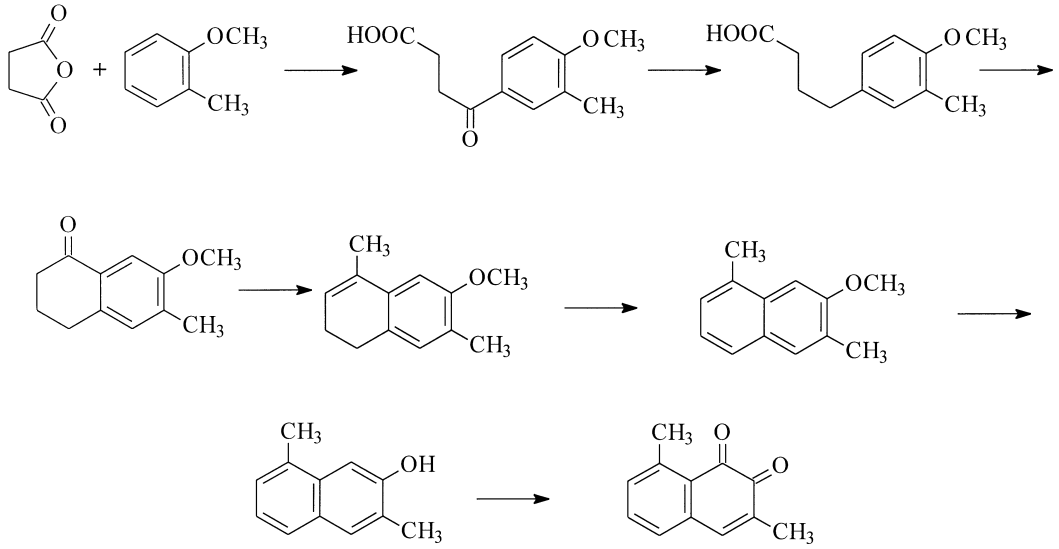
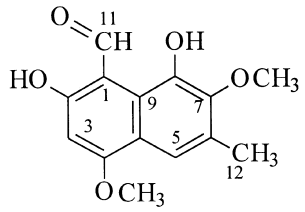


Fig. 1. Synthesis of *o*-hibiscanone (see Experimental section for reagents and product fields).

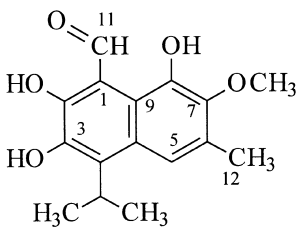
262.0846; Diff. 0.5 ppm). A fragment ion at *m*/*z* 244 indicated the loss of H_2O which is characteristic of sesquiterpenoid aldehydes found in other members of the Malvaceae (e.g., hemigossypol [1] (Scheme 1) and raimondal [6] (Scheme 3) from *Gossypium*). This fragmentation requires the presence of an aldehyde group at C-1 and hydroxyl groups at C-2 and C-8. Hibiscanal also gave a significant peak at *m*/*z* 260 (9%) which was shown by high resolution mass measurement to be $C_{14}H_{12}O_5$ (Calcd. 260.06847; Found 260.06847; Diff. 0.01 ppm).

The ^{1}H-NMR spectrum of hibiscanal (Table 1) showed a low field aldehyde proton at δ 10.16; and a chelated *ortho*-hydroxy proton appeared at δ 12.77. Two aromatic methoxyl groups (δ 3.89 and 4.02) and a methyl group (δ 2.32) were also evident. Aromatic proton singlets were observed at δ 6.72 and 7.57. The ^{1}H-^{1}H COSY spectrum showed a correlation between the methyl group (δ 2.32) and the aromatic proton at δ 7.57. This establishes that this aromatic proton is adjacent to the methyl group.

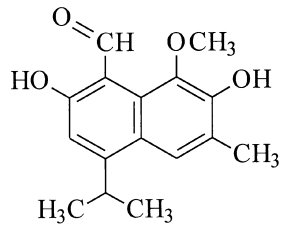
The ^{13}C-NMR spectrum (Table 2) showed 14 carbon atoms. An inverse ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) spectrum established the attachment of protons to the specific carbons in the naphthalene ring as shown in Table 2.



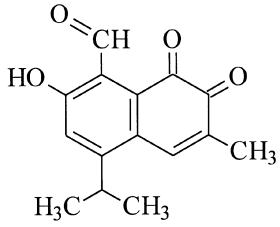
Hibiscanal
(*Hibiscus cannabinus*)



Raimondal
(*Gossypium*)



Isohemigossypol-1-methyl Ether
(*Bombax*)



Hibiscoquinone A
(*Hibiscus*)

Scheme 3. Comparison of hibiscanal structure with those of related terpenoids in other species of the Malvaceae.

Table 2. ^{1}H-NMR and ^{13}C-NMR chemical shifts of hibiscanal, raimondal, and 2,4-dihydroxyacetophenone

			Raimondal	2,4-Dihydroxyacetophenone
	H*	C	C	C
C#	δ	δ	δ	δ
1		110.8	111.4	113.9
2		166.1	166.1	165.1
3	6.72^{s}	94.9	141.9	103.5
4		171.2	134.3	165.1
5	7.57^{q}	115.3	117.3	108.6
6		130.9	127.0	133.9
7		149.3	142.8	
8		148.4	144.3	
9		125.2	115.0	
10		119.5	125.0	
11	10.19^{s}	190.8	199.0	
12	2.32^{d}	16.6	16.5	
13			27.7	
14			20.1	
15			20.1	
C_4-OCH_3	4.02^{s}	59.4		
C_7-OCH_3	3.19^{s}	57.3	60.9	
C_2-OH	12.77^{s}			

*s = singlet, q = quartet (0–8 Hz); d = doublet (0.8 Hz)

The specific point of attachment on the naphthalene ring of the protons and most of the groups was established by an inverse heteronuclear multiple bond connectivity (HMBC) experiment (Fig. 2). Thus, the aldehyde showed long-range correlations with the signal at δ 110.9 and δ 125.2. This established positions C-1 and C-9, respectively, in the naphthalene ring. The unique chemical shift of the signal at δ 94.9 indicated it was located between two carbons attached to oxygen moieties. The proton attached to this carbon (δ 6.72) showed long-range correlations to signals at δ 110.9 (C-1) and δ 119.4, and to two oxygenated atoms δ 165.8 and δ 171.2. The HMBC experiment showed that the signal at δ 171.2 is attached to an O-CH_3

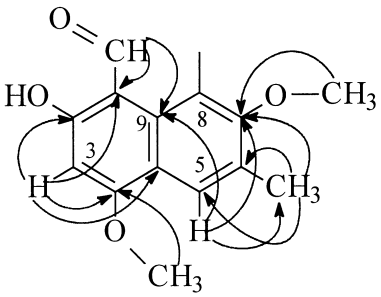


Fig. 2. HMBC correlation of hibiscanal.

group and thus the signal δ 165.8 must be C-2 and the signal at δ 119.4 must be C-10.

The shifts for carbons -1 to -4 of hibiscanal are in general agreement with C-1, C-2, C-3, and C-4 in 2,4-dihydroxyacetophenone [23] (Table 2). Substituents on the second naphthalene ring were established as follows. The HMBC experiment showed the proton at δ 7.57 was correlated to the signals at δ 125.2 (C-9) and δ 149.3 (C-OCH_3) and thus *meta* to these carbons. Thus, this proton is attached to either C-5 or C-7. The protons on the aromatic methyl group showed long range coupling to carbons at chemical shifts of δ 130.9, 115.2 (C-H) and 149.3 (C-OCH_3). Thus, the aromatic methyl group must be attached to the carbon at δ 130.9 with the carbons at δ 115.2 and 149.3 located on either side. The remaining ring carbon (i.e., C-8) which showed no long-range correlation was assigned to the signal at δ 148.4. The chemical shift of C-5, C-6, C-7, and C-8 in hibiscanal are very similar to those in raimondal (Table 2; Fig. 3) and thus are assigned as indicated. Oxygen substitution in this ring is also in agreement with that of *o*-hibiscanone.

Toxicities of phytoalexins to fungal pathogens

Both hibiscanal and *o*-hibiscanone are toxic to the fungal wilt pathogens *V. dahliae* and *F.o.v.* (Figs 3 and 4) *Verticillium dahliae* is more sensitive than *F.o.v.* to both compounds. Hibiscanal had ED_{50} values of 25.8 μg/ml and 36.5 μg/ml against *V. dahliae* and *F.o.v.*, respectively. *o*-Hibiscanone had significantly lower ED_{50} values toward these organisms (1.2 μg/ml for *V. dahliae* and 1.1 μg/ml for *F.o.v.*). *o*-Hibiscanone killed all propagules of *V. dahliae* and *F.o.v.* at 8 and 12 μg/ml, respectively. As shown in Fig. 5, *o*-hibiscanone was twice as toxic to *V. dahliae* as mansonone C which differs only by the addition of an isopropyl group at C-5 (Scheme 2).

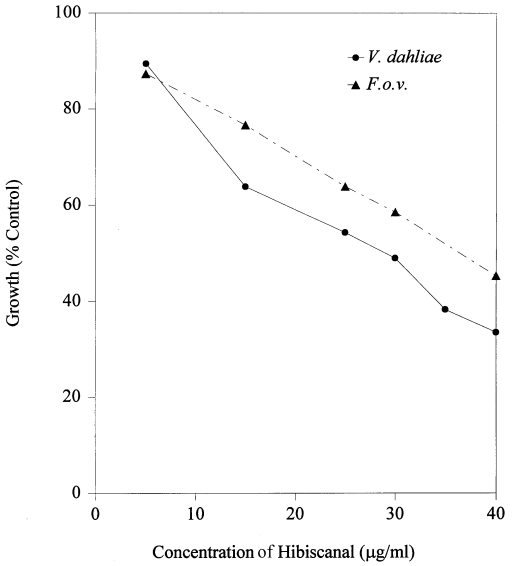


Fig. 3. Toxicity of hibiscanal to *Verticillium dahliae* and *Fusarium oxysporum* f. sp. *vasinfectum*. Values for each concentration are means of 6 replications from each of 2–4 experiments.

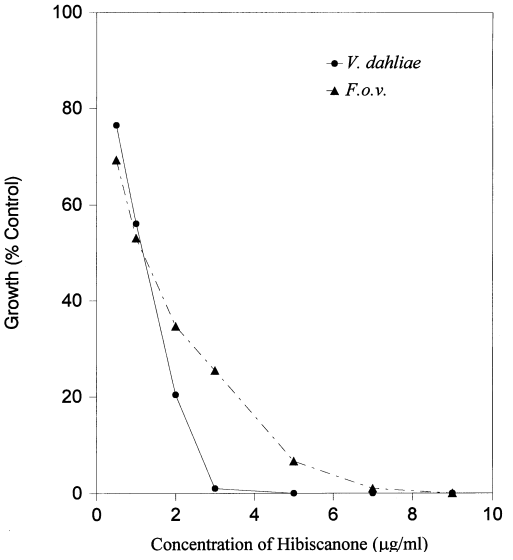


Fig. 4. Toxicity of *o*-hibiscanone to *Verticillium dahliae* and *Fusarium oxysporum* f. sp. *vasinfectum*. Values for each concentration are means of 6 replications from each of 2–4 experiments. Propagules of *V. dahliae* and *F.o.v.* were killed at 8 and 12 μg/ml, respectively.

DISCUSSION

Hibiscanal and *o*-hibiscanone function as phytoalexins synthesized by kenaf cells in response to infection by fungal wilt pathogens. In separate studies (Bell and Stipanovic, unpublished) we have shown that these compounds are secreted into infected xylem vessels beginning about 24 hr after inoculation and accumulate to toxic levels by 72 hr after inoculation. They do not accumulate in H_2O-inoculated plants. Because they are synthesized in response to stress and accumulate to fungitoxic levels they are phytoalexins.

We propose that the prefix hibiscan- be used to name the trinorcadalenes of *Hibiscus cannabinus* to distinguish them from the hibiscones (cadinenes) and

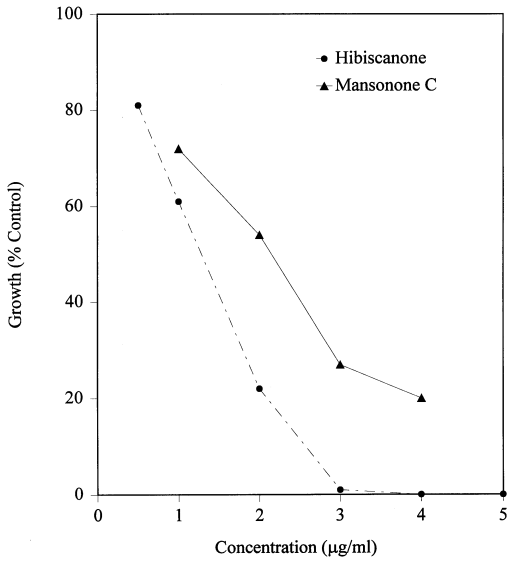


Fig. 5. Comparison of the toxicity of *o*-hibiscanone and mansonone C to *Verticillium dahliae* isolate V44.

hibisquinones (cadalenes) isolated from heartwood of other *Hibiscus* species [24, 25]. As shown in Schemes 2 and 3, the compounds from kenaf apparently differ from other terpenes found in malvaceous plants [24–28] because the isopropyl group, normally present in the cadalene ring, is absent from the naphthalene ring of the trinorcadalenes. In other species of the Malvaceae the terpenes apparently are derived from a cadinene, for example δ-cadinene in *Gossypium* [11, 12], and the entire carbon structure is maintained during synthesis of the biologically active terpenoids. Kenaf apparently produces a unique enzyme that allows removal of the isopropyl carbons from the cadinene ring, or even before ring closure. Precursor feeding studies are being used to test this possibility.

Several observations suggest that *o*-hibiscanone and hibiscanal may be responsible for the high level of resistance of kenaf to wilt pathogens. The ability to synthesize trinorcadalenes appears to be unique to kenaf among species of the Malvaceae. *o*-Hibiscanone is more toxic to *V. dahliae* than is mansonone C (Fig. 5), which is produced by other species of the Malvaceae [27, 28]. Yet, these compounds are identical in structure (Scheme 2), except for the isopropyl group. This suggests that the removal of the isopropyl group has improved the toxicity of the terpenoid *o*-naphthoquinones to wilt pathogens. Dumas et al [29] found that the mansonones A, C, D, E, F, and G also varied significantly in toxicity to the fungal wilt pathogen *Ceratocystis ulmi* due to small differences in the structure of these terpenoid *o*-quinones. *o*-Hibiscanone also is considerably more toxic to *V. dahliae* than dHG, the most potent phytoalexin produced by *Gossypium* species against wilt pathogens [22]. dHG has an ED_{50} value of 3.5 μg/ml against *V. dahliae* and kills all propagules at 15 μg/ml. In comparison, *o*-hibiscanone has an ED_{50} value of 1.18 μg/ml and kills all propagules at 8 μg/ml. Thus, *o*-hibiscanone is more toxic than any of the known phytoalexins in the Malvaceae. If the biosynthetic pathway, key enzymes and gene control of *o*-hibiscanone can be deciphered, it may be possible using recombinant DNA technology to transfer the necessary genes from kenaf to cotton in order to allow synthesis of the trinorcadalene phytoalexins and improve the resistance of cotton to wilt pathogens.

EXPERIMENTAL

General

Mass spectra were obtained on a double-focusing VG 70–250 EHF spectrometer with a VG 11–250 data system by direct probe using electron impact to generate ions. ^{1}H- and ^{13}C-NMR were obtained on a Bruker ARX-300 spectrometer in $CDCl_3$; all carbon assignments were confirmed by heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) experiments.

Pathogens

Verticillium dahliae Kleb. strains V-44 and V-76 (cotton defoliating isolates) and PH and TS2 (cotton nondefoliating isolates), were cultured on PDA for 7 day at 25°. Conidia were harvested in sterile distilled H_2O and filtered through Whatman No. 4 filter paper to remove mycelia. Spores were washed twice by centrifugation, resuspended in H_2O, and adjusted to *ca.* 10^7 spores/ml (*A*.050 at 600 nm for *V. dahliae* [7]). An isolate of *Fusarium oxysporum* Schlecht f. sp. *vasinfectum* Atk. Sny. & Hans. (*F.o.v.*; American Type Culture Collection 46644) from cotton in California was cultured and prepared as described for *V. dahliae*.

Growth and inoculation of kenaf

Kenaf, cv. Everglade, plants were grown in the field at College Station, Texas. When the stems were 4–5 month old, they were cut into 20 cm sections, and the bark (cortical tissue) tissue was removed to expose the cambial tissue of the stele. These stele sections were immediately dipped into a mixture of conidial suspensions prepared from the four *V. dahliae* strains, and were incubated over moist towels in plastic storage containers placed in large plastic bags without sealing to assure aeration. The containers were kept in darkness at room temperature for 72 hr. Non-inoculated plant steles served as the control.

Extraction of phytoalexins

The inoculated stele sections were cut into *ca.* 2 cm pieces and put into a 2 l flask with Me_2CO (5 ml/g fresh tissue) and shaken for about 5 min. Then, the extract was filtered, using a Whatman No. 42 filter paper to remove plant tissue. This extraction was per-

formed twice. The Me_2CO was evaporated from the extract in a rotary evaporator at 30°. The remaining aqueous suspension was mixed with an equal volume of EtOAc in a separatory funnel, and the H_2O phase was discarded. The EtOAc extract was kept in a refrigerator for further use. The extract from non-inoculated kenaf stele tissue was used as control.

Purification of phytoalexins by column chromatography

The extract was chromatographed on a 38×2.5 cm column of SilicAR CC-4 (Mallinckrodt, Inc.). The concentrated extract was dried onto a few grams of the Si gel and then placed at the top of the column which was eluted with 50 ml of each 0, 5, 10, 15, 20, 30, 40 and 100 percent Me_2CO in cyclohexane. 50 ml fractions were collected separately in flasks and dried on a rotary evaporator. Antifungal activities of each fraction were tested using the TLC bioassay.

TLC bioasssays for phytoalexins

Test samples were spotted on Baker Silica Gel G/HR TLC plates that were developed with solvent system 4 (see below). The plates were dried at room temp. in a fume hood, and then hot PDA medium was sprayed on the plates and allowed to solidify. Spore suspension (10^7 spores/ml) of *V. dahliae* isolate V-44 or *F.o.v.* then was sprayed on the TLC plate. Plates were incubated over moist towels in glass trays covered by thin plastic wrap. They were kept in the dark at room temp. (22–25°) for 2 days. Plates then were observed for fungal growth and inhibitory zones.

TLC purification of phytoalexins

TLC plates (20×20 cm) were prepared with Baker Silica Gel G/HR (0.5 mm). Developing solvents were: system 1, cyclohexane-EtOAc (60 : 40); system 2, hexane-Me_2CO (65 : 35); system 3, toluene-MeOH (95 : 5); and system 4, $CHCl_3$-Me_2CO-HCO_2H (84 : 15 : 1). Inhibitory compounds were located by visible colors or fluorescences under UV (365 nm). Zones containing inhibitory compounds were scraped from plates with a razor blade immediately after development. Compounds were eluted from silica gel with 40% Me_2CO in cyclohexane, dried and dissolved in EtOAc. Developing solvent systems 1–4 were used successively until antifungal compounds were pure. Compounds were detected by their visible colors, fluorescence under 365 nm UV, and reaction with phloroglucinol reagent (5% phloroglucinol in EtOH:conc. HCL, 1/1, freshly mixed) and $FeCl_3$-$K_3Fe(CN)_6$ reagent (a 1 : 1 mixture of 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$). All isolation procedures were modifications of methods used for cotton phytoalexins [1].

Characterization of o-hibiscanone.

o-Hibiscanone was obtained as red crystals from Me_2CO:hexane, mp 145–146°. UV: $\lambda EtOH_{max}$ nm (ϵ) 203 (22,650), 253 (27,600), 355 (2,700), 419 (3,100); IR: ν_{max}^{KBr} cm^{-1} 1670, 1657; MS: *m/z* (%) 188 (1.5, $[M]^+ + 2$), 186 (2.3, $[M]^+$), 159 (17), 158 (100, $[M]^+$-CO), 157 (16), 130 (22), 129 (36), 128 (24), 127 (11), 115 (54); ^{1}H-NMR ($CDCl_3$): δ 1.98 (3H, *bs*, C3-CH_3), 2.60 (3H, *s*, C8-CH_3), 7.05 (1H, *d*, $J = 7.6$ Hz, C5-H), 7.14 (1H, *bs*, C4-H), 7.15 (1H, *d*, $J = 7.6$ Hz, C7-H), 7.39 (1H, *t*, $J = 7.6$ Hz, C6-H); ^{13}C-NMR ($CDCl_3$): δ 15.3 (C3-CH_3), 22.8 (C8-CH_3), 127.8 (C5), 128.1 (C9), 133.6 (C7), 134.7 (C6), 135.8 (C3), 136.3 (C10), 142.6 (C4), 145.3 (C8), 181.0 (C1), 181.4 (C2).

X-ray crystallographic identification of o-hibiscanone

o-Hibiscanone crystallized in the monoclinic space group $P2_1/n$ with cell constants a = 9.065 (4)Å, b = 10.897 (6)Å, c = 18.802 (9) Å. The data were calculated on a Nicolet R3m/v X-ray defractometer with MoK$\propto$(0.710073 Å) radiation for $4.0° \leq 20 \leq 50.0°$ [w(Wyckoff) scans, $-10 \leq h \leq 10$, $0 \leq \kappa \leq 12$, $0 \leq \ell$, ≤ 22] at 193°K. Scan width, on ω, for the data collection was 0.60°, with a variable scan rate of 1.50 to 14.65° min^{-1}. A total of 1893 unique reflections, with $|I| \geq 2.0o$ I, were used for structure solution. The structure was solved by direct Methods [30]. Full matrix least-squares anisotropic refinement for all non-hydrogen atoms yielded $R = 0.075$, w$R = 0.078$ and $S = 2.18$ at convergence. The final difference Fourier map was featureless with $rho_{max} = 0.39$ and $rho_{min} = -0.39$ e Å^{-3}. Full details of the X-ray analysis will be provided upon request.

Characterization of hibiscanal

Hibiscanal was obtained as red crystals from Me_2CO:hexane, mp 225–226°. MS: *m/z* (%) 262 (7.6), 260 (9), 245 (25), 244 (100, $[M]^+$-H_2O), 229 (34, 244-CH_3), 213 (14), 201 (15, 229-CO); UV: λ_{max}^{EtOH} nm (ϵ) 402 (0.8050), 348 (5,500), 285 (17,400); ^{1}H-NMR and ^{13}C-NMR spectra are given in Table 2.

Biological activity of phytoalexins

The toxicities of compounds inhibitory to *V. dahliae* (V-44) and *F.o.v.* were quantitatively determined using a direct turbidimetric bioassay. The buffered nutrient solution (pH 6.3) was composed of 150 mM Na-K-Pi buffer containing 30 mM $NaNO_3$, 1 mM $MgSO_4.7H_2O$ and 115 mM D-glucose. Phytoalexins were dissolved in DMSO. The solutions were diluted with nutrient solution to give a final concentration of 2% DMSO. The toxicities of the compounds to *V. dahliae* (V-44) and *F.o.v.* were assayed by adding various concentrations of the phytoalexins to conidia (10^6 spores/ml) in nutrient solution. The assay mixtures (200 μl/well) were incubated in a 96-well cluster plate.

Each concentration of a compound was replicated X6 for each experiment. Plates were incubated for 40 hr at 25° in darkness, and optical densities were read directly on a Molecular Devices Precision Microplate Reader at 550 nm. Each experiment was repeated 2–4 times. Assay medium with 2% DMSO served as the control.

Preparation of 3-(4-*methoxy*-3-*methylbenzoyl*)-*propionic acid*

Succinic anhydride (110 g) and *o*-methylanisole (122 g) were dissolved in nitrobenzene (500 ml) under dry conditions and cooled to −15° in an ice-salt bath. Dry $AlCl_3$ (294 g) was added to the stirred soln over a period of 1 hr. The soln was warmed to 0° over 3 hr, and then slowly to room temp. with continued stirring for 40 hr. The reaction mixture was poured onto 1.2 kg of ice and 336 ml of 6N HCl. The white ppt. was removed by filtration and set aside. The filtrate was extracted with Et_2O (800 ml), and the organic phase was washed with 10% NaOH (300 ml). The alkaline solution was acidified with 6N HCl. The ppt. was removed by filtration and combined with the ppt. from above. After washing with H_2O, the ppt. was refluxed 3 hr with 20% NH_4OH (4.5 l). The soln was decolorized with activated carbon and filtered. The filtered soln was cooled in ice and acidified with HCl. The ppt. was filtered and washed with H_2O to provide 195 g of white crystals: mps 150.5–152°, lit. 152° [31]; ^{1}H-NMR ($CDCl_3$) δ 2.77 (2H, *t*, J = 6.6 Hz, C2), 2.22 (3H, *s*, CH_3), 3.24 (2H, *t*, J = 6.6 Hz, C3), 3.68 (3H, *s*, OCH_3), 6.83 (1H, *d*, J = 8.6 Hz, C5′), 7.77 (1H, *d*, J = 2.5 Hz, C2′), 7.81 (^{1}H, *dd*, J = 8.6 Hz, 2.5 Hz, C6′); ^{13}C-NMR ($CDCl_3$) δ 16.2 (CH_3), 28.2 (C2), 32.7 (C3), 55.2 (OCH_3), 109.2 (C5′), 126.8 (C3′), 128.2 (C6′), 129.0 (C1′), 130.7 (C2′), 161.9 (C4′), 178.8 (C1), 196.7 (CO).

Preparation of 1-(4-*methoxy*-3-*methylphenyl*)-*butyric acid*

Zinc (78 g), HgCl (7.8 g), H_2O (130 ml) and conc. HCl (4 ml) were mixed for 8 min. The solution was decanted and H_2O (49 ml), conc. HCl (114 ml), toluene (65 ml), and 3-(4-methoxy-3-methylbenzoyl)-propionic acid (40 g) were combined. The mixture was refluxed for 20 hr during which conc. HCl (32 ml) was added X3 at 6 hr intervals. The soln was cooled to room temp., H_2O (130 ml) was added, and the organic layer was separated. The H_2O layer was extracted with Et_2O (X3, 50 ml). The organic extracts were combined, washed with H_2O and placed over dry Na_2SO_4. The solvent was evaporated, and the product distilled under vacuum (bp, 167°/1mm Hg). The product (22.6 g) solidified, mp 57.5–59°; ^{1}H-NMR ($CDCl_3$): δ 1.93 (2H, *q*, J = 7.5 Hz, C3), 2.21 (3H, *s*, CH_3), 2.37 (2H, *d*, J = 7.5 Hz, C2), 2.59 (2H, *d*, J = 7.5 Hz, C4), 3.81 (3H, *s*, OCH_3), 6.75 (1H, *d*, J = 8.9 Hz, C5′), 6.96 (1H, *bs*, C2′), 6.97 (1H, *bd*, J = 8.9 Hz, C6′); ^{13}C-NMR ($CDCl_3$): δ 16.2 (CH_3), 26.4 (C3), 33.3 (C2), 34.1 (C4), 55.3 (OCH_3), 109.9 (C5′), 126.5 (C3′), 126.5 (C6′), 130.8 (C2′), 132.8 (C1′), 156.1 (C4′), 180.2 (C1).

Preparation of 7-*methoxy*-6-*methyl*-1-*tetralone*

4-(4-Methoxy-3-methylphenyl) butyric acid (22 g) was mixed with polyphosphoric acid (130 g) under N_2 and heated at 85° for 6 hr. The mixture was cooled, carefully diluted with ice and H_2O (200 ml) and extracted with Et_2O (500 ml). The Et_2O soln was washed with H_2O and placed over dry Na_2SO_4. The solvent was evaporated, and the crude product was distilled (bp, 148–152°/2.5 mm Hg). The product (17.3 g) solidified, mp 44–45°; UV: λ_{max}^{EtOH} nm (ϵ) 206 (17,000) 225 (20,300), 262 (12,500) 320 (4,400); IR: ν_{max}^{KBr} cm^{-1} 1674, 1630, 1566, 1495; ^{1}H-NMR ($CDCl_3$): δ 2.06 (2H, quin, C3), 2.21 (3H, *s*, CH_3), 2.57 (2H, *t*, J = 6.2 Hz, C2), 2.82 (2H, *t*, J = 6.1 Hz, C4), 3.82 (3H, *s*, OCH_3), 6.98 (1H, *s*, C5), 7.41 (1H, *s*, C8); ^{13}C-NMR ($CDCl_3$): δ 16.5 (CH_3), 23.6 (C3), 28.7 (C4), 38.8 (C2), 55.4 (OCH_3), 106.7 (C8), 130.8 (C5), 131.3 (C9), 133.7 (C6), 137 (C10), 156.5 (C7), 198.2 (C1).

Preparation of 1,2-*Dihydro*-6-*methoxy*-4,7-*dimethylnaphthalene*

7-Methoxy-6-methyl-1-tetralone (17.1 g) was dissolved in 1 l of Et_2O under N_2. A 3M solution of methyl magnesium bromide (131 ml) was added dropwise over 1 hr. The soln was refluxed for 2 hr, and then cooled. An aq. soln of NH_4Cl (23 g in 64 ml H_2O) was added dropwise. The Et_2O soln was separated, washed with H_2O and evaporated to dryness. The crude product (19.08 g) was dissolved in C_6H_6 (700 ml) and a catalytic amount of *p*-toluenesulfonic acid was added. The soln was refluxed for 1 hr under N_2 using a Dean-Stark trap. After cooling, Et_2O (300 ml) was added, washed with 5% $NaHCO_3$ soln(250 ml), dried over Na_2SO_4, and evaporated. The crude product was distilled (bp, 115°/0.45 mm Hg) to provide 13.6 g of a light yellow oil; ^{1}H-NMR ($CDCl_3$): δ 2.08 (3H, *bq*, J = 1.77 Hz, Cl-CH_3), 2.23 (2H, *m*, C4), 2.25 (3H, *bs*, C6-CH_3), 2.68 (2H, *t*, J = 8.2 Hz, C4), 3.86 (3H, *s*, OCH_3), 5.84 (1H, *bt*, C2), 6.76 (1H, *s*, C8), 6.93 (1H, *bs*, C5); ^{13}C-NMR ($CDCl_3$): δ 15.8 (C6-CH_3), 19.4 (Cl-CH_3), 23.5 (C3), 27.4 (C4), 55.6 (OCH_3), 105.3 (C8), 124.7 (C6), 124.8 (C2), 128.1 (C10), 129.7 (C5), 132.1 (C1), 134.4 (C9), 156.3 (C7).

Preparation of 7-*methoxy*-1,6-*dimethylnaphthalene*

1,2-Dihydro-6-methoxy-4,7-dimethyl-naphthalene (13.2 g) was dissolved in decalin (100 ml). Palladium on carbon (10%, 2 g) was added and the mixture was refluxed for 18 hr under N_2. The catalyst was removed by filtration, and most of the decalin was removed by distillation; the product (11.4 g) was crystallized from cyclohexane, mp 69–70°; UV: λ_{max}^{EtOH} nm (ϵ) 231 (46,500), 268 (3,300), 277 (3,700), 287 (3,100), 313

(1,100), 327 (1,500); IR: ν_{max}^{KBr} cm^{-1} 1629, 1599, 1502, 1447; ^{1}H-NMR (Me_2CO-d_6): δ symbol: 2.32 (3H, *bs*, C6-CH_3), 2.61 (3H, *bs*, Cl-CH_3), 3.96 (3H, *s*, OCH_3), 7.18 (1H, *t*, $J=7.0$ Hz, C3), 7.22 (1H, *bd*, C2), 7.23 (1H, *bs*, C8), 7.57 (1H, *d*, $J=7.0$ Hz, C4), 7.59 (1H, *bs*, C5); ^{13}C-NMR (Me_2CO-d_6): δ 16.8 (C6-CH_3), 19.6 (Cl-CH_3); 55.6 (OCH_3), 102 (C8), 124.0 (C3), 126.1 (C4), 128.5 (C6), 129.9 (C1), 130.2 (C5), 133.4 (C10), 133.5 (C9), 157.7 (C7).

Preparation of 3,8-*dimethyl*-2-*naphthol*

7-Methoxy-1,6-dimethylnaphthalene (10 g) was dissolved in dry CH_2Cl_2 (120 ml). The soln was cooled to $-80°$ in a dry ice/Me_2CO bath. A 1 M solution of BBr_3 in CH_2Cl_2 (60 ml) was added. The soln was allowed to warm slowly to room temp. The soln was diluted with Et_2O (100 ml) and washed with 5% $NaHCO_3$ soln followed by H_2O. After drying over Na_2SO_4, the solvent was evaporated. The naphthol (2.1 g) was recrystallized from hexane, mp, 90.5–91°; UV: λ_{max}^{EtOH} nm (ϵ) 231 (58,800), 271 (3,800), 281 (4,900), 292 (4,000), 318 (1,900), 331 (2,000); IR: ν_{max}^{KBr} cm^{-1} 3352, 1629, 1532, 1440, 1235; ^{1}H-NMR (Me_2CO-d_6): δ 2.37 (3H, *bs*, C3-CH_3), 2.52 (3H, *bs*, C8-CH_3), 7.12 (1H, *t*, $J=7.7$ Hz, C6), 7.16 (1H, *bd*, $J=7.7$ Hz, C7), 7.30 (1H, *bs*, C1), 7.54 (1H, *d*, $J=7.7$ Hz, C5), 7.58 (1H, *bs*, C4); ^{13}C-NMR (Me_2CO-d_6): δ 16.7 (C3-CH_3), 19.4 (C8-CH_3), 106.1 (C1), 123.3 (C6), 126.2 (C5), 126.6 (C7), 127.6 (C3), 129.7 (C10), 130.4 (C4), 132.5 (C8), 133.7 (C9), 155.2 (C2).

Preparation of 3,8-*dimethyl*-1,2-*naphthoquinone* (*o-hibiscanone*)

Benzene selenic anhydride (298 mg) was added to dry THF (16.5 ml). A soln of 3,8-dimethyl-1-naphthol (133 mg) in dry THF (8 ml) was added dropwise over 15 min. The reaction was heated to 50° and held at that temp. for 1 hr. After cooling, Et_2O (25 ml) was added to the reaction. The soln was washed twice with brine (50% satd soln, 20 ml) and dried over Na_2SO_4. The solvent was evaporated, and the product (107 mg) was crystallized from C_6H_6/hexane, mp 147–148°, mixed 145–146°.

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REFERENCES

1. Bell, A. A., Stipanovic, R. D., Howell, C. R. and Fryxell, P. A., *Phytochemistry*, 1975, **14**, 225.
2. Stipanovic, R. D., Bell, A. A. and Howell, C. R., *Phytochemistry*, 1975, **14**, 2861.
3. Bell, A. A., in *Challenging the Future*: *Proceedings of the World Cotton Research Conference*-1, ed. G. A. Constable and N. W. Forrester. CSIRO, Melbourne, Australia, 1995, p. 225.
4. Bell, A. A., Stipanovic, R. D., O'Brien, D. H. and Fryxell, P. A., *Phytochemistry*, 1978, **17**, 1297.
5. Bell, A. A., Stipanovic, R. D., Mace, M. E. and Kohel, R. J., *Recent Advances in Phytochemistry*, 1994, **28**, 231.
6. Stipanovic, R. D., Bell, A. A. and O'Brien, D. H., *Phytochemistry*, 1980, **19**, 1735.
7. Bell, A. A., *Phytopathology*, 1969, **59**, 1119.
8. Cui, Y., Bell, A. A., Puckhaber, L., Joost, O. and Magill, C., *Molecular Plant-Microbe Interactions*. 1998, submitted.
9. Mace, M. E., *Physiological Plant Pathology*, 1985, **12**, 1.
10. Stipanovic, R. D., Mace, M. E., Altman, D. W. and Bell, A. A., in *Biological Active Natural Products*, ACS Symposium Series Number 380, ed. H. G. Cutler. American Chemical Society, Washington, D.C., 1988, p. 262.
11. Alchanati, I., Patel, J. A. A., Liu, J., Benedict, C. R., Stipanovic, R. D., Bell, A. A., Cui, Y. and Magill, C. W., *Phytochemistry*. 1997, in press.
12. Chen, X. Y., Chen, Y., Heinstein, P. and Davisson, V. J., *Archives of Biochemistry and Biophysics*, 1996, **324**, 255.
13. Joost, O., Bianchini, G., Bell, A. A., Benedict, C. R. and Magill, C. W., *Molecular Plant-Microbe Interactions*, 1995, **8**, 880.
14. Bell, A. A. and Stipanovic, R. D., *Mycopathologia*, 1978, **65**, 91.
15. Mace, M. E., Stipanovic, R. D. and Bell, A. A., *Physiological Plant Pathology*, 1985, **26**, 209.
16. Mace, M. E., Stipanovic, R. D. and Bell, A. A., *Pesticide Biochemistry and Physiology*, 1990, **36**, 79.
17. Mace, M. E., Stipanovic, R. D. and Bell, A. A., *Natural Toxins*, 1993, **1**, 294.
18. Zhang, J., Mace, M. E., Stipanovic, R. D. and Bell, A. A., *Journal of Phytopathology*, 1993, **139**, 247.
19. Fryxell, P. A., *The Natural History of the Cotton Tribe*. Texas A&M University Press, College Station, Texas, 1979, p. 245.
20. Kohel, R. J. and Bell, A. A., *Journal of Heredity*. 1997, in press.
21. Idessis V. F., in *Cotton Wilt*, ed. M. V. Mukhamedzhanov. U. S. Department of Commerce, National Technical Information Service, Springfield, Va. 22151, 1966 (translated 1972), p. 61.
22. Stipanovic, R. D., Puckhaber, L. S. and Bell, A. A., in *Synthesis and Chemistry of New and Potential Agrochemicals*. eds. D. R. Baker, J. G. Fenyes, G. S. Basarab, D. A. Hunt. American Chemical Society, Washington, D.C., 1997, in press.

23. Dhami, K. S. and Stothers, J. B., *Canadian Journal of Chemistry*, 1965, **43**, 479–498.
24. Ferreira, M. A., King, T. J., Ali, S. and Thomson, R. H., *J. C. S. Perkin I*, 1980, 249.
25. Ali, S., Singh, P. and Thomson, R. H., *J. C. S. Perkin I*, 1980, 257.
26. Sankaram, A. V. B., Reddy, N. S. and Shoolery, J. N., *Phytochemistry*, 1981, **20**, 1877.
27. Letcher, R. M. and Shirley, I. M., *Phytochemistry*, 1992, **31**, 4171.
28. Neelakantan, S., Rajagopalan, V. and Raman, P. V., *Indian Journal of Chemistry*, 1983, **22H**, 95.
29. Dumas, M. T., Strunz, G. M., Hubbes, M. and Jeng, R. S., *European Journal of Forestry Pathology*, 1986, **16**, 217.
30. Sheldirck, G. M. *SHELXTL-Plus* (*rev*. 4.11) *User Manual*. Siemens Analytical X-ray Instruments Inc., Madison, WI, 1992.
31. Violland, R., Violland-Duperret, N., Pacheco, H., Trouiller, G. and Leblane, A., *Chimie Therapeutique*, 1971, **6**, 196.